Regulation of Plasminogen Activation

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Academic Dissertation

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CONTENT

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following orginal articles referred to in the text by Roman numericals I-IV:

I Myöhänen HT, Stephens RW, Hedman K, Tapiovaara H, Rønne E, Høyer-Hansen G, Danø K, Vaheri A. Distribution and lateral mobility of the urokinase-receptor complex at the cell surface. The Journal of Histochemistry and Cytochemistry 41:1291-1301, 1993.

II Sirén V, Myöhänen H, Vaheri A, Immonen I. Transforming growth factor beta induces urokinase receptor expression in cultured retinal pigment epithelial cells. Ophthalmic Research 31**:** 184-191, 1999.

III Myöhänen H, Virtanen I, Vaheri A. Elimination of hydrocortisone from the medium enables tissue plasminogen activator gene expression by normal and immortalized nonmalignant human epithelial cells. Biological Chemistry 382: 1563-1573, 2001.

IV Myöhänen H, Sirén V, Suomalainen I, Wahlström T, Vaheri A. Tissue plasminogen activator is expressed strongly in normal and dysplastic stratified squamous epithelium of the human uterine cervix. Submitted, 2003.

ABBREVIATIONS

AP-1	activating protein-1
ARMD	age-related macular degeneration
ATCH	adrenocorticotrophin
α_2M	α_2-macroglobulin
ATF	amino-terminal fragment
BEAS2B	human bronchial epithelial cells (adeno-12/SV-40 virus-transformed)
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
DMBA	7,12-dimethylbenzantracene
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
FITC	fluorescein isothiocyanate
FPR	formyl peptide receptor
FPRL1	FPR-like receptor-1
GR	glucocorticoid receptor
GRE	glucocorticoid response element
HCE16/3	human cervical epithelial cells (HPV-16 virus-transformed)
HES	human embryonic skin fibroblast
HGF/SF	hepatocyte growth factor/scatter factor
IFN-α/γ	interferon-α/γ
IGF	insulin-like growth factor
IGFBP-4	insulin-like growth factor-binding protein-4
IL-1/4	interleukin-1 or -4
LRP	low-density lipoprotein-related protein
Ly-6 /(TAP)	lymphocyte antigen-6/ T-cell-activating protein
MMP	matrix metalloproteinase
MT-MMP	membrane-type matrix metalloproteinase
NF-κB	nuclear factor-kappa B
NHBE	normal human bronchial epithelial cells
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
PA	plasminogen activation
PAI-1	plasminogen activator inhibitor-1
PAI-2	plasminogen activator inhibitor-2
PDGF	platelet-derived growth factor
PEA3	polyomavirus enhancer A-binding protein-3
PMA	12-tetradecanoylphorbol-13-acetate

PVR	proliferative vitreoretinopathy
RA	retinoic acid
RD	rhabdomyosarcoma cells
RPE	retinal pigment epithelium
SDS	sodium dodecyl sulfate
SMC	smooth muscle cell
suPAR	soluble urokinase receptor plasminogen activator
T_3	triiodothyronine
TGF-β	transforming growth factor-β
TIMP	tissue inhibitor of matrix metalloproteinase
tPA	tissue plasminogen activator
TRITC	tetramethyl rhodamine isothiocyanate
uPA	urokinase plasminogen activator
uPAR	urokinase plasminogen activator receptor
VEGF	vascular endothelial growth factor
VN	vitronectin

ABSTRACT

The main components of plasminogen activation include plasminogen, tissue plasminogen activator (tPA), urokinase plasminogen activator (uPA), urokinase plasminogen activator receptor (uPAR), and plasminogen activator inhibitors-1 and -2 (PAI-1, PAI-2).

uPA and its receptor uPAR are known to be important in cell migration/adhesion. In these studies, we show that uPAR was anchored in focal contacts in a uPA-dependent manner, whereas in its unoccupied form uPAR appeared diffusely on the cell surface. Moreover, $\alpha_v\beta_3$-integrin was found in close proximity to uPAR in the focal contacts.

We also studied the effect of transforming growth factor-β (TGF-β) and interferon γ (IFN-γ) retinal pigment epithelial cells (RPE) and observed that TGF-β increased uPAR expression.

tPA has been considered to be mainly a fibrinolytic enzyme. We observed that in human uterine cervical epithelial cells immortalized with HPV-16 (human papillomavirus type 16) DNA, the tPA activity levels were very low or even undetectable due to an inhibitory effect of hydrocortisone in the growth medium. Withdrawal of hydrocortisone increased tPA mRNA expression, which seemed to be a later event than uPA mRNA expression. tPA activity levels increased dramatically at 72 hours when hydrocortisone was eliminated from the growth medium. Elimination of hydrocortisone also increased tPA and uPA activity levels in two other nonmalignant human epithelial cells.

Based on results from in vitro observations, the presence of tPA was examined in normal, precancerous, and malignant epithelial tissue lesions by immunohistochemistry. In addition, in situ hybridization was used to demostrate tPA mRNA expression. Immunohistochemistry revealed that tPA was expressed at high levels in normal, condylomatous, and dysplastic squamous epithelium. Expression of tPA mRNA was restricted to the proliferating basal and parabasal cell layers, whereas tPA protein synthesis became evident in the next cell layers. tPA immunostaining in cancer cells was mostly negative or weak in adenocarcinoma and strong in epidermoid carcinoma. We detected tPA mRNA in adenocarcinoma but not in epidermoid carcinoma. In epidermoid carcinoma, the surrounding stromal cells were positive.

REVIEW OF THE LITERATURE

PLASMINOGEN ACTIVATION

Serine proteinases belong to a family of proteinases that hydrolyze peptide bonds in proteins. They contain the amino acid serine in their active center. Enzymes of the plasminogen activator cascade also belong to this family. The end product of the plasminogen activation cascade is plasmin, a proteolytic enzyme with a broad substrate affinity. Historically, plasminogen, plasmin, and plasminogen activators have been associated with fibrinolysis (Astrup, 1966; Christman et al., 1977; Danø, 1985). Already in the early 1890s, fibrin was known to be digested and not dissolved in blood (Macfarlane and Biggs, 1948). In 1933, Tillett and Garner demonstrated that hemolytic streptococcal broth could dissolve a normal human fibrin clot. The active factor originating from the streptococcal broth was called fibrinolysin. Later, Milstone (1941) noticed that when the fibrin preparation was sufficiently pure, fibrinolysis failed to occur or occurred very slowly. However, addition of the human serum euglobulin fraction once again enabled fibrinolysis. Milstone revealed that human serum contains a "lytic factor", and this lytic factor together with bacterial fibrinolysin could lyse fibrin clots. Christensen (1945) and Christensen and Macleod (1945) discovered that the lytic factor and serum proteinase are one and the same enzyme occurring in a precursor form and that both can be activated by fibrinolysin. They demonstrated that fibrinolysis is a proteolytic activity and that this enzyme was capable of cleaving fibrin, fibrinogen, casein, and gelatin. They suggested a revised nomenclature to avoid confusion with other proteinases in the blood/serum. The serum proteinase and the active form of the lytic factor were named plasmin, and the precursor form was called plasminogen. Streptococcal fibrinolysin they named streptokinase (Christensen, 1945; Christensen and MacLeod, 1945; Astrup and Permin, 1948).

How does plasminogen activation occur? Animal tissues were found to contain a fibrinolytic activator (Astrup and Permin, 1947) which could "transform" profibrinolysin (plasminogen) into fibrinolysin (plasmin). In the 1947 scheme, this transformation was shown to occur spontaneously, following the treatment of serum with chloroform, streptokinase, or tissue activator. A major obstacle was that these activators could not be purified, since, for example, tissue activator had a strong

adherence (affinity) to cellular components, hindering the purification process. Although fibrin-cleaving activity was detected in urine nearly 100 years ago (Astrup, 1956), the identification took place many decades later. This substance was called urokinase since it was isolated from human and dog urine (Williams, 1951; Astrup and Sterndorff, 1952; Sobel et al., 1952). Plasminogen activator from urine and blood was added to the scheme of plasminogen activation, and trypsin was also shown to activate plasminogen (Astrup, 1956). The mechanism of conversion of plasminogen to plasmin was discovered by Robbins et al. in 1967.

CONTRIBUTING FACTORS IN PLASMINOGEN ACTIVATION

Plasminogen

Plasminogen is a single-chain glycoprotein with a molecular weight of approximately 92 kDa. Plasminogen is synthesized mainly in the liver (Raum et al., 1980), and this inactive form circulates in the vasculature but is also found ubiquitously in all body fluids. Secreted plasminogen is a precursor that is cleaved by plasminogen activators at a single site, the Agr_{561}-Val_{562} bond (Robbins et al., 1967; Petersen et al., 1990), to yield a two-chain plasmin held together by two disulfide bonds (Sottrup-Jensen et al., 1978). The structural features of the plasminogen molecule include an N-terminal (A-chain) component, which has a pre-activation peptide (from 1-77), followed by five tandem structures called kringle domains. Kringle domains participate in binding to fibrin and to the cell surface (Ponting et al., 1992). In its B-chain (carboxyl-terminal region) lies the catalytic domain with the His-Asp-Ser characteristic triad of serine proteinases (Petersen et al., 1990; Parry et al., 2000).

The proenzyme plasminogen exists in the circulation with Glu (glutamic acid) amino acid in its NH_2-terminus, and cleavage of the N-terminal peptide produces Lys-terminal plasminogen, which is more easily activated by plasminogen activators (Fig. 1). Removal of the terminal Glu is not, however, a prerequisite for the activation of plasminogen. The N-terminal peptide is probably needed for the regulation of a conformational change and for the activation properties of the native molecule (Ponting et al., 1992). Plasminogen can also be cleaved with trypsin to activate it to plasmin and with elastase. Elastase cleavage of plasminogen yields two fragments: angiostatin including kringles 1-4 and miniplasmin consisting of the serine protease domain and

kringle 5 (Parry et al., 2000) (Fig. 1). Angiostatin has been shown to inhibit angiogenesis (Pepper, 2001).

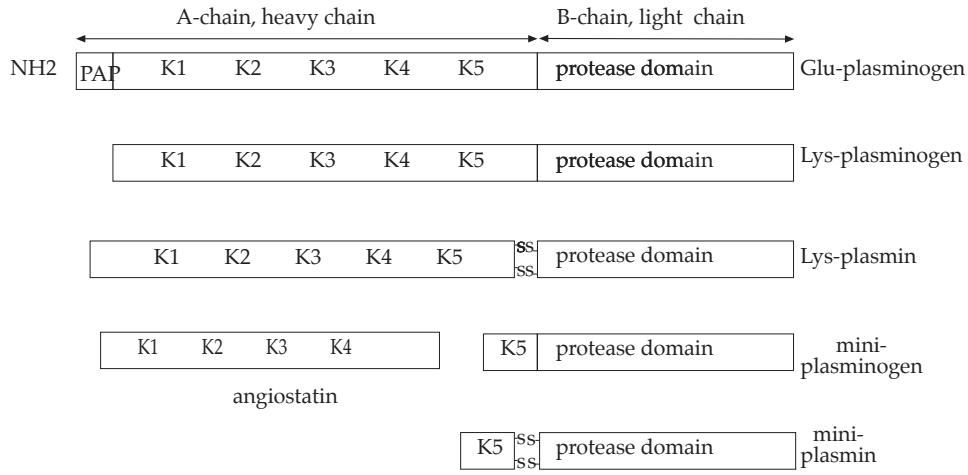

Figure 1. Schematic diacram of plasminogen and its cleavage products. PAP = pre-activation peptide, K=kringle, Glu=glutamatic acid, Lys=lysine.

Tissue plasminogen activator (tPA)

The mRNA for tPA is approximately 2.7 kb and encodes a glycoprotein with a molecular weight of about 70 kDa (Pennica et al., 1983; Fisher et al., 1985). tPA protein is secreted as a single-chain glycoprotein. Similarly to uPA, tPA can be cleaved by plasmin to a two-chain form held together by a single disulfide bond (Table 1). The tertiary structure of tPA differs from that of uPA. The N-terminal (A-chain or heavy-chain) region has a finger domain followed by a growth factor domain, and two kringle domains. The carboxyl-terminal (B-chain or light-chain) region contains a serine protease domain (Ny et al., 1984). Unlike uPA, single-chain tPA has significant activity (Tachias and Madison, 1996). Binding of tPA to fibrin enhances plasminogen activation markedly. The finger domain and kringle domain are important in binding of tPA to fibrin (Collen, 1999). Endothelial cells in particular produce tPA (Levin and del Zoppo, 1994; Levin et al., 1997), as do human keratinocytes, melanocytes, and neurons (Chen et al., 1993; Bizik et al., 1996; Teesalu et al., 2002). Of neoplastic cells, tPA can be produced

by melanoma, neuroblastoma, ovarian cancer, breast cancer, and pancreatic cancer cells (Rijken and Collen, 1981; Amin et al., 1987; Neuman et al., 1989; Paciucci et al., 1998).

Urokinase plasminogen activator (uPA)

The urokinase mRNA of 2.5 kb (Verde et al., 1984) encodes a single-chain glycoprotein with a molecular weight of 53 kDa (Lesuk et al., 1965; Wun et al., 1982a, 1982b). The enzyme is activated by cleavage of a single peptide bond. This cleavage can be catalyzed by plasmin, for example (Wun et al., 1982b; Collen et al., 1986; Tapiovaara et al., 1993). Active urokinase is a two-chain form held together by a single disulfide bond (Table 1). The tertiary structure of uPA is composed of three different domains. The N-terminal A-chain (light chain) includes a growth factor domain followed by one kringle domain (Blasi et al., 1987, 1988). These together are known as the amino-terminal fragment (ATF) (1-135 amino acids) (Stoppelli et al., 1985). The receptor-binding site has been mapped to the growth factor domain of the ATF-fragment (Appella et al., 1987). In the carboxyl-terminal region, also known as B-chain (heavy chain), uPA has a serine protease domain. The two chains are linked with a connecting peptide (tPA does not have it). Active two-chain uPA is also referred to as high molecular weight (HMW) urokinase. Additional cleavage between amino acids 134-135 separates the light chain (ATF-fragment) and the heavy chain containing the active serine protease domain. This cleavage has also been shown to occur with plasmin (Blasi et al., 1987, 1988). Urokinase is secreted by many types of cancer cells, including breast, colon, ovary, gastric, cervix, endometrium, bladder, kidney, and brain tumor tissues, in higher amounts than by the corresponding normal tissues (Andreasen et al., 1997). While the cells producing uPA in cancer tissue remain unidentified, in colon carcinoma urokinase has been established to be secreted mainly by the cancer cells themselves (Berger, 2002).

Table 1. Structural features of tPA and uPA.

	tPA	uPA
mRNA molecular weight	2.7 kb 70 kDa	2.5 kb 53 kDa
A-chain	finger domain growth factor domain two kringles (heavy chain)	growth factor domain one kringle (ATF-fragment, light chain)
B-chain	protease domain (light chain)	protease domain (heavy chain)
connecting peptide	not included	included
zymogenity	low	high

Receptors for plasminogen activators

Cell membrane-bound high-affinity receptors for tissue plasminogen activator have not been identified. Yet, tPA binds to many proteins, and fibrin is by far the most efficient binding protein. In at least one respect, fibrin can be said to act as a receptor for tPA; in the presence of fibrin, tPA-catalyzed plasminogen activation occurs at least 100-fold more effectively than in its absence (Hoylaerts et al., 1982; Collen, 1999). If not a true receptor, it is at least a cofactor for tPA. Other tPA candidate receptors include mannose-6-phosphate/IGF II receptor, annexin II, and amphoterin (Otter et al., 1991; Parkkinen and Rauvala, 1991; Hajjar et al., 1994). In addition, tPA binds to extracellular matrix components such as laminin and fibronectin (Salonen et al., 1984, 1985).

uPA has high-affinity binding sites on the cell surface (Stoppelli et al., 1985, 1986 Vassalli et al., 1985). Urokinase plasminogen activator receptor (uPAR), originally purified from several different cell lines, has a molecular weight of approximately 55 kDa (Nielsen, 1988). The receptor showed specific binding of urokinase and the urokinase ATF-fragment. The mRNA for uPAR is 1.4 kb (Roldan et al., 1990). Binding of uPA to its receptor is species-specific, i.e. human uPA does not bind to murine uPAR and murine uPA does not bind to human uPAR (Estreicher et al., 1989). Fully processed uPAR is heavily glycosylated (Behrendt et al., 1990). In its carboxyl-terminal region, uPAR has a glycosyl-phosphatidylinositol moiety (GPI-anchor) by which uPAR is integrated into the outer leaflet of the cell membrane (Ploug et al., 1991). The tertiary structure of uPAR is formed by three homologous repeats known as domains 1, 2, and 3 (D1, D2, D3) (Behrendt et al., 1991; Ploug et al., 1993). Based on its protein structure, uPAR belongs to the glycolipid-anchored Ly-6 superfamily of proteins (Ploug and Ellis,

1994). The ligand-binding site is located in domain 1 (D1), which is also the first repeat starting from the amino-terminus (Behrendt et al., 1991). Purified or cell membrane-bound uPAR is cleaved either partially or totally (Behrendt et al., 1991; Ploug et al., 1991; Høyer-Hansen et al., 1992), and it therefore exists in soluble form. High levels of soluble uPAR (suPAR) can be measured, for example, in patients with acute myeloid leukemia (Mustjoki et al., 2000). uPAR is expressed at elevated levels in breast colon, gastric, and certain lung carcinoma tissues (Andreasen et al., 1997). Disagreement exist between different laboratories as to which cells produce uPAR, whether it is produced by stromal cells such as macrophages or by cancer cells in breast and colon carcinomas tissues (Mazar, 2001).

Plasminogen activator inhibitors

PAI-1 and PAI-2

The two known physiological inhibitors for uPA and tPA are plasminogen activator inhibitors-1 and -2 (PAI-1 and PAI-2, respectively). PAI-1 and PAI-2 belong to the gene family of serine protease inhibitors called serpins (Ny et al., 1986; Antalis et al., 1988;k Ye et al., 1989). Both are glycoproteins. The molecular weight of PAI-1 is about 54 kDa (Andreasen et al., 1986a). PAI-1 has two mRNAs (2.4 and 3.2 kb), which is thought to be due to alternative polyadenylation sites. Serpins can adopt a variety of conformations under physiological conditions. PAI-1 can exist in its native inhibitory form, in an inactive, latent form, in complexes with proteinases, and in a cleaved substrate form (Lawrence, 1997, 2000). In addition, PAI-1 can form complexes with vitronectin, while simultaneously retaining its activity (Salonen et al., 1989). Cultured endothelial cells as well as many other cultured cells secrete PAI-1 (Hekman and Loskutoff, 1985). In the fibrosarcoma cell line HT-1080 (Nielsen et al., 1986), PAI-1 is also bound to the substratum of the cells (Pöllänen et al., 1987). PAI-1 is the major inhibitor of tPA in circulation. Platelets contain a large pool of PAI-1, mostly in an inactive form (Andreasen et al., 1990).

PAI-1 forms complexes both with single-chain and two-chain tPA and with two-chain uPA (Andreasen et al., 1986b). However, PAI-1 has also been shown to form a reversible complex with single-chain uPA (Manchanda and Schwartz, 1995). Receptor-bound active urokinase is inhibited by PAI-1 and PAI-2 (Cubellis, 1989; Stephens et al., 1989; Ellis et al., 1990), which leads to the inhibition of plasminogen activation.

Based on the amino acid and gene structure, PAI-2 belongs to a subgroup of the serpin superfamily, ovalbumin-type serpins (Ye et al., 1989; Silverman et al., 2001). Its gene is transcribed to a 2.0-kb mRNA (Ye et al., 1989). PAI-2 protein exists in two forms: in a nonglycosylated intracellular form and in a glycosylated, secreted extracellular form (Belin, 1992). PAI-2 can also be detected as a polymer intracellularly (Mikus and Ny, 1996). The molecular weight of the intracellular form is about 42 kDa and that of the secreted form 60 kDa. PAI-2 inhibits uPA rapidly, but tPA very slowly (Andreasen et al., 1990). PAI-2 is expressed as an inflammatory response in macrophages and also in keratinocytes under certain conditions (Belin, 1992; Chen et al., 1993).

α2-antiplasmin and α2-macroglobulin

The serpin inhibitor α_2-antiplasmin is the primary inhibitor of plasmin (Wiman and Collen, 1978). It forms a complex with plasmin by occupying the plasmin kringle domains, i.e. the lysine-binding sites. It is a fast inhibitor of free plasmin in circulation and in solution. When plasmin/plasminogen is bound to the cell surface, its lysine-binding sites are occupied, and therefore, α_2-antiplasmin acts more slowly (Ponting et al., 1992).

α_2-macroglobulin is found in circulation in vast quantities (Sottrup-Jensen, 1989) It is a large glycoprotein which can trap proteinases and their inhibitor complexes. α_2-macroglobulin complexes are internalized by means of low-density lipoprotein-related receptor (LRP) (Borth, 1992). α_2-macroglobulin binds plasminogen activators and their inhibitor complexes as well as plasmin. Cell-bound plasmin is protected from α_2-macroglobulin.

PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL ROLES OF PLASMINOGEN ACTIVATION

Despite uPA and tPA being impressively similar in structure and having common inhibitors and physiological substrates, their physiological roles are quite distinct. Traditionally, the role of tPA has been as a mediator of fibrinolysis in vasculature (Lijnen and Collen, 1991; Collen, 1999). Urokinase is strongly linked to cell migration, especially in cancer cells (Andreasen et al., 1997). Thus far, more than one target for tPA/plasmin has been found in neuronal tissues (Chen and Strikland, 1997; Wu et al., 2000; Traynelis and Lipton, 2001). The functional role of the PAIs is no longer simply to

inhibit overexpressed plasminogen activators; PAI-1 also participates in cell adhesion via the adhesion protein vitronectin (Loskutoff et al., 1999) and in angiogenesis (Bajou et al., 1998), and PAI-2 has an unidentified role in the regulation of cell death (Silverman et al., 2001).

Factors influencing plasminogen activation

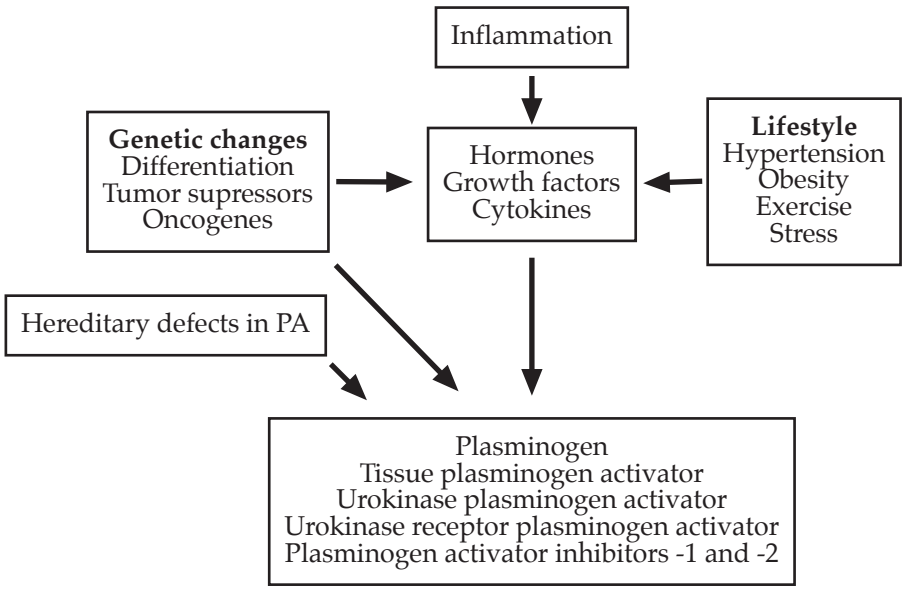

Figure 2. Factors influencing increasing and decreasing expression of plasminogen activation (PA) components. Structural defects of PA components disturb the balance of plasminogen activation.

Consequences of plasminogen activation

The discovery that plasmin solubilizes the fibrin network after its polymerization led to the utilization of plasminogen activators as thrombolytic agents in cardiovascular diseases and ischemic stroke (Lijnen and Collen, 1991; Collen, 1999). In knock-out mice studies, plasminogen and its activators deficiency has been demonstrated to cause severe thrombosis (Carmeliet et al., 1994; Bugge et al., 1995). uPAR-uPA participate in

fibrinolysis, but extravascularly (Idell, 2002). Plasmin is necessary in wound healing (Bugge et al., 1996; Rømer et al., 1996) and matrix metalloproteinases (MMPs) are needed to complete the wound healing process (Lund et al., 1999).

In physiological or pathophysiological conditions, PA and MMPs act in concert. Proteolytic processing of the structural hemidesmosome protein laminin-5 is an example of one such target protein. Recent in vitro studies have addressed plasmin and tPA regulating the proteolytic processing of the α_3-chain of laminin-5, which results in the assembly of stabilized hemidesmosome structures and cessation of cell migration (Goldfinger et al., 1998). The same group demonstrated that uPA, plasmin, and integrin ligation with uPAR was involved in the processing of the laminin-5 α_3-chain, thus reducing cell motility (Ghosh et al., 2000). Cleavage of the laminin-5 γ_2-chain by MMP-2 induces cell migration (Giannelli et al., 1997), although MT1-MMP was later shown to be mainly responsible for the truncated form of the laminin-5 γ_2-chain with or without MMP-2 (Koshikawa et al., 2000). Interestingly, uPAR and the truncated form of the laminin-5 γ_2-chain are coexpressed in colon adenocarcinomas in front of invasive cancer cell islands (Pyke et al., 1995). Moreover, the overexpression of uPA and uPAR (bitransgenic mouse) in basal mouse keratinocytes increased plasminogen activation and cooperated in pathogenic proteolysis, which led to the partly disruption of hemidesmosomes and epidermal blistering (Zhou et al., 2000). In the same study, MMP-9 and MMP-2 were shown to be activated only in bitransgenic mice. However, the proteolytic conversion of plasminogen to plasmin can occur without uPAR (Carmeliet et al., 1998). Plasmin can activate several matrix metalloproteinases in vivo; for example, during aneurysm formation MMP-3, -9, -12, and -13 are activated (Carmeliet et al., 1997).

uPA-uPAR complex formation on the cell surface has proteolytic, cell migratory, adhesive, and more recently characterized chemotactic effects. The cleavage of uPAR between domain 1 (D1) and domain 2 (D2) exposes an epitope fragment on the side of D2D3 uPAR which is chemotactic (Fazioli et al., 1997). The binding of uPA to uPAR seems to be needed to unmask the chemotactic uPAR sequence. Whether the cleavage occurs by uPA or other proteinases in vivo remains to be seen (Fazioli et al., 1997; Koolwijk et al., 2001). It is not clear whether the D2D3 soluble form, the membrane-attached form, or both induce the chemotactic effect in vivo (Blasi and Carmeliet, 2002). The D2D3 sequence of uPAR interacts with the chemotactic formyl peptide receptor (FPR)/FPR-like receptor-1 (FPRL1) (Resnati et al., 2002). The presence of the two

activators in the joints of rheumatoid arthritis patients seems to have opposing effects on disease severity: tPA has a protective role and uPA is deleterious (Yang et al., 2001; Cook et al., 2002). This is possibly based on tPA-mediated fibrin clearance and uPA-mediated recruitment of inflammatory cells and cytokines in arthritic joints.

Plasmin has the capacity to release and activate growth factors, hormones, and proteases such as transforming growth factor-β (TGF-β) (Lyons et al., 1990; Taipale et al., 1992), fibroblast growth factor (FGF) (Saksela and Rifkin, 1990), insulin-like growth factor-binding protein-4 (IGFBP-4) (Remacle-Bonnet et al., 1997), and latent collagenases (O'Grady et al., 1981; Mazzieri et al., 1997). In vitro, plasmin converts proinsulin, adrenocorticotrophin (ACTH) (Granelli-Piperno and Reich, 1983), and interleukin-1β (IL-1β) (Matsushima et al., 1986) in their active forms. In addition, uPA has been shown to activate hepatocyte growth factor (HGF) in vitro (Naldini et al., 1995). Plasmin can also activate latent TGF-β in a reaction where uPAR facilitates this activation on the cell surface via bound uPA (Odekon et al., 1994).

The plasminogen activation system is involved in pathological conditions of the human eye. Retinal pigment epithelium (RPE) is composed of a single layer of cells between the retina and the choroid. Photoreceptors (rods and cones) of the retina are in contact with RPE. Bruch's membrane underlies the RPE. Thickening of Bruch's membrane is an early clinical sign of age-related macular degeneration (ARMD) (Ruberti et al., 2003), a severe form of which is choroidal neovascularization (CNV). In eye diseases such as ARMD and proliferative vitreoretinopathy (PVR) detachment of retinal pigment epithelial cells and their migration can occur (Glaser and Lemor, 1989; Elner, 2002). RPE cells secrete uPA and PAI-1 (Sirén et al., 1992). Retinal glial and retinal endothelial cells have also been shown to secrete tPA (Wileman et al., 2000; Schacke et al., 2002). Migration of RPE cells occurs in an uPAR-PAI-1 dependent manner (Elner, 2002). It is also noteworthy that the MMP inhibitor TIMP3 and PAI-1 have opposite effects on choroidal neovascularization; TIMP3 inhibits angiogenesis by blocking vascular endothelial growth factor (VEGF) from binding to its receptor (Qi et al., 2003) and PAI-1 enhances angiogenesis (Lambert et al., 2001).

Plasminogen activation and tumorigenesis

The connection between increased fibrinolytic activity and cancer was initially described more than 80 years ago. The pioneers Alex Carrel and Albert Fischer, among others, discovered that cancer cells continuously dissolve plasma clots in culture by proteolytic digestion (Danø, 1985). In the 1970s, the evidence was strengthened by plasminogen activators being produced in higher amounts in transformed cell lines (Unkeless et al., 1974). Plasminogen activation has been firmly linked to cancer cell invasion. Plasmin with its degrading capacity has been thought to facilitate tumor cells passage through barriers, such as basement membranes and interstitial connective tissue, and migration to distant body sites. The invasion of cancer cells is associated with the secretion of high amounts of uPA (Andreasen et al., 1997). An elevated level of uPA correlates with malignancy (Duffy, 2002). This has also been shown in the reverse. Human epidermoid carcinoma cells lose their malignant behavior if they are cultured for a prolonged time. This is accompanied by a number of changes, including reduced uPA synthesis (Ossowski and Belin, 1985). High levels of uPA and uPAR are related to poor prognosis, and it therefore came as a surprise that high PAI-1 levels are also a marker for negative prognosis in certain cancers (Schmitt et al., 1997).

Cancer cells have an enormous capacity to migrate. The mechanism, by which plasminogen activation facilitates cancer cell migration has been thought to be proteolysis of extracellular matrice alone. Recently, it has become clear that the situation is much more complex and that alternative routes exist within the plasminogen activation system. The components of the plasminogen activation cascade, namely uPA, uPAR, and PAI-1, take part in cell adhesion and migration. This function is partly independent of the proteolytic or inhibitory functions of the PA components. The first suggestion that PAI-1 plays a role in cell adhesion came from Ciambrone and McKeown-Longo (1990), who demonstrated that PAI-1 can disturb the interaction between vitronectin and its receptor. More recently, uPAR has been discovered to be a ligand for integrins (Tarui et al., 2001). In addition, PAI-1-channelled cell detachment is mediated by endocytosis of the uPA-uPAR-PAI-1-integrin complex (Czekay et al., 2003).

As already mentioned, High levels of uPA and unexpectedly PAI-1 are associated with poor prognosis in many cancers, including breast cancer (Schmitt et al., 1997; Duffy, 2002). The role of PAI-1 in cancer cells is unclear. It might be related to

angiogenesis in which PAI-1 seems to be involved. Alternatively it may be due to either the protease-inhibitory or the adhesive function of PAI-1 or both (Bajou et al., 2001; McMahon et al., 2001). Both the protease-inhibitory and the adhesive function via vitronectin/integrin participate in regulation of cancer metastasis (Praus et al., 2002).

Role of tPA in the brain

In 1981, tPA was demonstrated to be released at the neuronal growth cone (Krystosek and Seeds, 1981). The morphological differentiation of neuroblastoma cells was accompanied by tPA induction, also suggested to have a role in neuronal cell functions (Neuman et al., 1989). Involvement of plasmin(ogen) as well as tPA in brain function has been studied in knock-out mice. tPA participates in neuronal plasticity such as involvement in memory and learning activities (Madani et al., 1999). Moreover, tPA has been shown to operate in stress-induced neuronal plasticity (Fig. 2) (Pawlak et al., 2003). The corticosteroid hormones act in memory and learning progress as well as being involved in stress-caused processing of the neuronal network of the amygdala (Kloet et al., 1999). Mice deficient in tPA have higher stress tolerance and decreased neuronal remodeling within the amygdala. In addition, tPA-deficient mice show elevated and extended strength of corticosteroid levels after restraint stress (Pawlak et al., 2003). Regulation of hippocampal synaptic reorganization is facilitated by tPA both in a plasmin-dependent and -independent manner (Pawlak and Strickland, 2002). A proteoglycan is one target of plasmin-mediated cleavage in the central nervous system (Wu et al., 2000). tPA can also promote mossy fiber extension without its catalytic activity (Wu et al., 2000). tPA can also cause neuronal cell death (Tsirka et al., 1996). Plasmin is involved in hippocampal neuronal cell death and in the destruction of laminin via activation by tPA (Chen and Strikland, 1997).

The N-methyl-D-aspartate (NMDA) receptors (NMDAR) are classical memory and learning receptors. The NMDARs are glutamate-gated ion channels. Glutamate is the principal excitatory neurotransmitter in the mammalian central nervous system (Melbrum, 2000; Riedel et al., 2003). Activated presynaptic terminals release glutamate, which binds to NMDR in the plasma membrane of depolarized postsynaptic cells. Glutamate opens the NMDAR channel allowing Ca^{2+} (or Na^{+}, K^{+}) to enter the cell. Elevated Ca^{2+} acts as an intracellular mediator, activating for example enzymes. Excess of glutamate causes Ca^{2+} to enter cells and can result in neurotoxicity. When severe, it

leads to cell necrosis and in a milder form to cell apoptosis. Glutamate and its receptors play a critical role in pathological conditions such as cerebral ischemia, traumatic brain injury, and acute and chronic neurodegeneration. Patients with ischemic stroke are treated with tPA. While this treatment can cause neuronal cell damage, underlying the mechanism remains obscure. It has been demonstrated that tPA can augment neuronal cell destruction by directly cleaving the NMDA receptor (Nicole et al., 2001). tPA-cleaved truncated NMDAR forms are thought to allow excessive Ca^{2+} influx, leading to neurotoxicity and neuronal damage (Traynelis and Lipton, 2001).

Inactive tPA has a beneficial role in brain injury; without its proteolytic activity, it can activate microglial cells, which then further enhance their activation by releasing tPA (Rogove et al., 1999).

PAI-1 and PAI-2 in disorders

Disturbances in the amount of PAI-1 in plasma and its structural defects have been related to pathological conditions such as atherosclerosis (Wiman, 1995) and bleeding disorders (Diéval et al., 1991). The level of plasma PAI-1 is elevated in patients with cardiovascular and thromboembolic disease (Wiman, 1995), in those patients with diabetes (Banfi et al., 2001), and in obesity, which is a factor linked to diabetes (Samad et al., 1999) (Fig. 2). PAI-1 is also an acute-phase reactant and has been shown to be induced in mouse hepatic cells in response to tissue injury (Seki et al., 1999). The level of PAI-1 in tumor extracts is also an independent prognostic marker for recurrence-free survival of primary breast cancer (Hansen et al., 2003). Regardless of its linkage to many severe diseases, the origin of plasma PAI-1 remains unknown.

Elevated uPA, uPAR, and PAI-1 levels indicate an increased risk of developing metastasis in primary breast cancer, whereas elevated levels of PAI-2 suggest a favorable prognosis (Andreasen et al., 1997; Schmitt et al., 1997). The unusual intracellular occurrence of PAI-2 has long been a mystery. Cytosolic PAI-2 has been proposed to participate in host defense against viral infection. PAI-2 is also required for cell survival, since it inhibits TNF-α-induced apoptosis (Dickinson et al., 1995). However, in a study where PAI-2 protected cells from certain viral cytopathic effects, the reason was discovered to be that PAI-2 mediated interferon production and this led to expression of antiviral genes (Antalis et al., 1998). PAI-2 also has an unidentified role in the regulation of carcinogenesis caused by exposure to chemical carcinogens. High

expression of PAI-2 mRNA is induced rapidly by dioxin in human keratinocytes (Sutter et al., 1991). The overexpression of PAI-2 in transgenic mice had an enhancing effect on papilloma formation, some of which progressed to carcinoma, as studied by the multistage skin carcinogenesis model (Zhou et al., 2001). Moreover, as lesions progressed to invasive carcinoma, a change occurred in gene expression; PAI-2 was turned off and uPA turned on. Massive apoptosis in papillomas of control mice developed after the cessation of PMA application, while in transgenic mice limited apoptosis occurred. However, in this model, dimethylbenz(a)anthracene (DMBA) and PMA did not induce endogenous PAI-2 mRNA expression in control mice.

REGULATION OF PLASMINOGEN ACTIVATION

A key question in the plasminogen activation field has been about how the entire activation cascade is initiated. All enzymes in the PA system are secreted in proforms (plasminogen, uPA, and tPA). Single-chain (proenzyme) tPA has significantly higher intrinsic enzymatic activity than uPA (Andreasen et al., 1991; Renatus et al., 1997). In fact, there has been debate about whether uPA has any intrinsic enzymatic activity in its proenzyme form (Collen et al., 1986; Petersen et al., 1988). Some studies suggest that PAI-1 cannot bind and inhibit pro-urokinase (Andreasen et al., 1986b) and others claim that it can form reversible enzyme-inhibitor complexes (Manchanda and Schwartz, 1995). PAI-1, is, however, shown to behave as a substrate to its enzyme. After forming a complex (uPA-PAI-1), uPA is released in active form and PAI-1 in inactive form (Lawrence et al., 2000). Some reports also describe the proenzyme form of uPA as having intrinsic enzymatic activity (Lijnen et al., 1991). Activation of pro-urokinase or plasminogen can occur by kallikrein (List et al., 2000; Selvarajan et al., 2001). In addition, plasminogen, which circulates in closed conformation, will adopt an open conformation upon binding on fibrin (Ponting et al., 1992; Wang et al., 2000). Plasminogen itself undergoes an unusual conformational change when bound to 6-aminohexanoic acid (6-AHA) which mimics binding to fibrin (Mangel et al., 1990). In this open conformation, it is believed to be more readily activated.

Endocytosis

Endocytosis regulates the clearance of uPA and tPA from the cell surface, the circulation, and the extracellular space. Receptor-bound urokinase is most efficiently

internalized in complex with its inhibitor PAI-1 (Cubellis et al., 1990). This triad is endocytosed by means of the α_2-macroglobulin receptor/low-density lipoprotein receptor-related protein (α_2M/LRP, same as LRP) from the cell surface (Nyjkær et al., 1992; Conese et al., 1995; Hussain et al., 1999). Internalized uPAR is recycled to the cell surface (Nyjkær et al., 1997). The clearance of tPA-PAI (as well as of uPA-PAI-1) complexes from the circulation occurs via LRP in hepatic cells (Orth et al., 1992). uPAR is needed to present the uPA-PAI complex to α_2M/LRP (Olson et al., 1992). Such a receptor is not known for tPA.

Effects of hormones, growth factors, and cytokines on plasminogen activation

A retinoic acid receptor response element is located in the human tPA gene (Bulens et al., 1995). Retinoic acid induces tPA expression in microvascular endothelial, oral squamous carcinoma, and neuroblastoma cells (Tiberio et al., 1997; Lansink et al., 1998). More recently, a multihormone responsive enhancer element, which is activated by glucocorticoids, progesterone, androgens, and mineralocorticoids (Bulens et al., 1997), was identified in the tPA promoter, and it has four glucocorticoid receptor binding sites. The human uPA gene enhancer region contains PEA3/AP-1A and AP-1B sites which are pivotal for the induction of uPA gene transcription by phorbol 12-myristate 13-acetate (PMA) and epidermal growth factor (EGF) (Rørth et al., 1990; Nerlov et al., 1992). The PEA3/AP-1 transcription factors have been shown to bind in a cell type-specific manner on the uPA gene enhancer element (Nerlov et al., 1991). The human tPA gene contains AP-1 sites (Arts et al., 1997). uPA gene transcription is regulated by the family of nuclear factor-kappa B (NF-κB) transcription factor proteins (Novak et al., 1991; Newton et al., 1999; Wang et al., 1999).

Urokinase gene transcription repression is regulated by heterodimeric AP-1 transcription factors (De Cesare et al., 1995). PAI-1 has a glucocorticoid response element (GRE) site on its gene-regulating elements (van Zonneveld et al., 1988). In addition, an insulin response element has been mapped to the PAI-1 gene promoter region (Banfi et al., 2001).

The response of uPAR gene expression to transforming growth factor-β (TGF-β) varies strongly in different cell lines. This was tested in several human normal and neoplastic cell lines (Lund et al., 1991). For example, in lung carcinoma cells, TGF-β

enhanced uPAR mRNA. In addition, the secretion of uPA and PAI-1, but not of tPA, was affected by TGF-β (Lund et al., 1991).

TGF-β is a well-known stimulator of PAI-1 gene transcription (Laiho et al., 1987; Sawdey et al., 1989). The TGF-β-inducible transcription factors SMAD3 and SMAD 4 also bind to PAI-1 TGF-β response elements in the PAI-1 gene promoter (Dennler et al., 1998). TGF-β enhancement of PAI-1 expression can be repressed by glucocorticoids. This occurs with protein-protein interaction between GR and TGF-β response elements binding the proteins SMAD3 and SMAD4 (Song et al., 1999). TGF-β regulates the invasiveness of breast cancer cells by enhancing secretion of uPA, resulting in stimulation of plasmin formation. The amounts of uPAR, and PAI-1 are also increased. tPA levels by contrast, are decreased with TGF-β (Farina et al., 1998).

Addition of EGF to human epidermoid carcinoma (A431) cells was demonstrated to down-regulate epidermal growth receptors (EGFR) with a concomitant increase in plasminogen activators (Gross et al., 1983). Furthermore, the EGF-induced tPA activity has been shown to be hidden by PAI-1 in the A431 cell line (George et al., 1990). In a colon carcinoma cell line, EGF decreased uPA production preceding the uPAR increase. Insulin and transferrin had no effect on uPA and uPAR expression (Boyd, 1989). In human squamous cell carcinoma cells, addition of EGF and scatter factor (SF, HGF) had an inducing effect both on uPA and uPAR (Rosenthal et al., 1998). Nevertheless, MMPs induced invasion of these cells. Normal human uroepithelial cells produce tPA and uPA but neoplastic epithelia produce uPA alone. EGF had a slight effect on uPA expression in neoplastic uroepithelial cells. tPA production, by contrast, diminished after the cells reached, saturation. Addition of EGF had a considerable increasing effect on tPA expression in normal epithelium (Dubeau et al., 1988).

Neither Vascular endothelial growth factor (VEGF) nor basic fibroblastic growth factor (bFGF) alone affected tPA levels, but together they induced tPA in bovine endothelial cells. Other serum factors seem to be needed because the VEGF- and bFGF-induced tPA levels were not as high as in the presence of serum (Li and Keller, 2000). VEGF-B leads to increased expression of uPA as well as of PAI-1 (Olofsson et al., 1998). In mouse smooth muscle cells, the mitogenic or proliferative response by PDGF or bFGF was reduced in mice deficient of tPA or uPA. The effect of PDGF was shown to require the presence of tPA, and bFGF the presence of uPA. Both plasminogen activator mRNAs

were induced several-fold, uPA with bFGF and tPA with PDGF (Herbert et al., 1997). In pancreatic carcinoma cells, FGF2 regulates tPA and PAI-1 expression (Escaffit et al., 2000). In the prostate epithelial cell line, FGF7 induces invasion of cells accompanied by overexpression of MMP-1 and uPA (Ropiquet et al., 1999).

Table 2. Effect of growth factors, cytokines, and hormones on the plasminogen activation system.

Effector	Cells	tPA	uPA	uPAR	PAI-1
RA	Neuroblastoma	increase	no effect	no effect	no effect
	Oral squamous carcinoma cells	increase	no effect		
	Microvascular endothelial cells	increase	increase		
EGF	Epidermoid carcinoma (A431)	increase	increase		no effect
	Colon carcinoma cells		decrease	increase	
	Neoplastic uroepithelium cells	no effect	minor decrease		
	Normal uroepithelium cells	increase	increase		
	Squamous cell carcinoma (UM-SCC-1)	no effect	increase	increase	
HGF/SF	Squamous cell carcinoma (UM-SCC-1)	no effect	increase	increase	
TGF-β	Lung carcinoma (A549)	no effect	increase	increase	
	Breast cancer cells	decrease	increase	increase	
VEGF	Endothelia cells (serum needed)	increase			increase
dFGF	Endothelial cells (serum needed)	increase			increase
bFGF, FGF-2	Pancreatic carcinoma	increase			decrease
FGF7	Prostatic epithelial cells		increase		
IL-4	Vascular SMC (serum needed)	increase	no effect		decrease
IFN-γ	Vascular SMC	decrease	no effect		
	Monocytes			increase	
	Colon carcinoma cells (HTC116)			increase	
IFN-α	Colon carcinoma cells (HTC116)			increase	
PDGF	Vascular SMC	increase	no effect		
IL-1	Keratinocytes	increase			

Regulation of the plasminogen activation component by cytokines has mostly been studied in blood cells including monocytes/macrophages and endothelial in cells but also muscle and epithelial cells. Among the cytokines, interleukin-1β increased tPA production by the human keratinocytes cell line (Rox et al., 1996). In human aortic smooth muscle cells, IL-4 increased tPA antigen and IFN-γ decreased the effect of IL-4 in

the presence of serum. The presence of platelet-derived growth factor (PDGF) was also shown to have an effect on tPA antigen levels and thereby on muscle cell migration (Wang et al., 1995). The fibrinolytic potential was stimulated by LPS, TNF-α, and INF-γ on human endothelial cells (Arnman et al., 1995). Monocytes, which take part in inflammatory reactions, apparently respond to IFN-γ and TNF-α by increasing uPA binding on the cell surface; when combined, the two factors are strongly synergistic (Kirchheimer et al., 1988). IFN-α or -γ upregulates uPAR protein expression in the colon cancer cell line (Wu et al., 2002). Plasminogen is regulated by IL-6 (Jenkins et al., 1997). Furthermore, an IL-6 response element has been characterized on a murine plasminogen promoter sequence (Bannach et al., 2002).

Effect of hydrocortisone on plasminogen activation

The repressing and increasing effects of glucocorticoids are due to the relative impact of these steroids on different components of the PA system. They repress plasminogen activation by increasing inhibitor (e.g. PAI-1) synthesis (Bator et al., 1998) or by decreasing activator synthesis (Cwikel et al., 1984; Busso et al., 1987). In human HT-1080 fibrosarcoma cells, dexamethasone was demonstrated to increase tPA and PAI-1 synthesis and decrease uPA and PAI-2 synthesis, the net result being decreased PA activity in the cell culture medium (Medcalf et al., 1988). In addition, in HT-1080 cells, dexamethasone reduces the number of plasmin-binding sites on the cell surface (Pöllänen, 1989).

Glucocorticoids can also have the opposite effect on the synthesis of the two PA activators (Busso et al., 1986) without affecting inhibitors in the same cells. In human embryonic or tumor-derived carcinoma cell cultures, dexamethasone decreased plasminogen activator activity but had no repressive effect on six melanoma-derived cell cultures, which mainly produced tPA (Roblin and Young, 1980). In fact, dexamethasone increased tPA activity in these cultures. In rat hepatic cells, dexamethasone increased tPA gene transcription, and when combined with cAMP, tPA was upregulated further (Kathju et al., 1994). The increase in tPA activity by dexamethasone was also seen in rat granulosa cells under the influence of certain hormones and growth factors (Jia et al., 1990). However, in human ovarian carcinoma cells, dexamethasone decreased total plasminogen activator activity by 95% (Amin et al., 1987). The decrease in uPA activity preceded the effect on tPA activity. Moreover, there

was a marked effect on cell morphology by dexamethasone. In human embryonic lung cells, glucocorticoids have a suppressive effect on plasminogen activator synthesis (Rifkin, 1978).

One example of the impact of hydrocortisone on plasminogen activator(s) is given by tissue remodeling events such as mammary gland involution. Hydrocortisone inhibits mammary gland involution (Ossowski et al., 1979; Feng et al., 1995). uPA, as well as metalloproteinases, is strongly upregulated in mammary gland involution. In hydrocortisone-treated mice, the involution regresses, and the proteases (including uPA) are strongly repressed (Lund et al., 1996). Another example of tissue remodeling is provided by castrated rats. Prostate involution was accompanied by uPA and tPA upregulation, which was retarded by the administration of hydrocortisone, followed by downregulation of the uPA and tPA activities (especially the 30-kDa form of uPA) (Freeman et al., 1990). Benign and malignant prostate tissues were shown to contain both types of plasminogen activators; albeit more tPA than uPA. The presence of hydrocortisone in prostate tissue organ culture led to a marked decrease of plasminogen activators (Camiolo et al., 1984).

Early reports demonstrate that the administration of corticotrophin significantly decreases the elevated fibrinolysis seen in patients with liver cirrhosis (Kwaan et al., 1956). In rat tongue organ culture, the squamous tongue epithelium was shown to produce tPA. The addition of hydrocortisone to the culture medium led to a marked repression of tPA activity (Wünschmann-Henderson and Astrup, 1974). In the same study, hydrocortisone-treated cultures were observed to have better tissue integrity, although this could not been confirmed to be the result of the decrease in tPA. Maintenance of mammary epithelial cell differentiation is partly due to extracellular matrix turnover caused by the inhibitory effect of glucocorticoids (Casey et al., 2000).

Human keratinocytes secrete both plasminogen activators and PAI-1 in vitro (Jensen et al., 1990), and their secretion depends on the differentiation state of the epithelial cell. Furthermore, immunohistological staining of uPA and tPA in organotypic explants showed that tPA and uPA were localized in different cell layers of stratified keratinocytes (Chen et al., 1993). Hydrocortisone has also been studied in murine keratinocytes, where it was found to have an increasing effect on PAI-1 mRNA and antigen levels, but not on tPA and uPA mRNA (Bator et al., 1998). In this system, plasminogen activation was mainly suppressed due to the increased PAI-1 production.

Table 3. Effect of hydrocortisone or dexamethasone on plasminogen activation.

Cells	PA-activity	tPA	uPA	PAI-1	PAI-2
Fibrosarcoma-HT1080	decrease	increase	decrease	increase	decrease
Melanoma cell lines:					
Malme-3M	no effect	no effect			
RPMI 8252	no effect	no effect			
SK-MEL-2	no effect	no effect			
SK-MEL-26	no effect	no effect			
SK-MEL-27	no effect	no effect			
MeWo	no effect	no effect			
Embryonic lung cells	inhibit				
Embryonal kidney cells (HEK)	inhibit		decrease		
Uveal melanocytes	inhibit		decrease		
Renal carcinoma (Caki-1)	inhibit		decrease		
Renal carcinoma (Caki-2)	inhibit				
Lung adenocarcinoma (Calu-3)	inhibit				
Mammary carcinoma (MDA-MB-231)		decrease	decrease	increase	
Rat hepatoma cells	decrease	no effect		increase	
Ovarian carcinoma (OVCA 433)	decrease	decrease	decrease		
Rat ovarian granulosa cell		increase			
Murine keratinocytes	decrease	no effect	no effect	increase	
Mammary gland involution	inhibit	decrease	decrease		
Rat prostate gland involution	inhibit	decrease	decrease	no effect	
Neoplastic prostate organ culture		decrease	decrease		
Benign prostate organ culture		decrease	decrease		
Rat organ tongue culture	inhibit	decrease			
Patients with cirrhosis, plasma levels	decrease				

INTERACTION OF PLASMINOGEN ACTIVATION COMPONENTS WITH ADHESION MOLECULES

In vitro, urokinase is localized on the cell surface at focal contacts, tips of microspikes, cell-cell contacts, and lamellipodia (Pöllänen et al., 1987, 1988). uPAR is GPI-anchored (Ploug et al., 1991), which prompted researchers to look for associated molecules. Finding an explanation for those uPA-uPAR-related cellular events that were independent of uPA's proteolytic activity (e.g. cell migration caused by the formation of uPA-uPAR complexes) was of particular interest (Gudewicz and Gilboa, 1987; Fibbi et al., 1988; Del Rosso et al., 1990).

Earlier findings of PAI-1 being deposited on the substratum of cell cultures (Pöllänen et al., 1987) and forming complexes with vitronectin while retaining its activity (Salonen et al., 1989) suggested that PAI-1 is more than an inhibitor. The first hints that PAI-1 participated in cell adhesion came from Ciambrone and McKeown-Longo (1990), who found that the addition of PAI-1 antibodies disturbs cell adhesion. This was shown to be due to the disruption of vitronectin-dependent adhesion. They later, demonstrated that the localization of uPA depends on the cell substratum. In cells on growing vitronectin-coated surfaces, uPA was localized in focal contact-like areas but not on fibronectin, on which uPA was evenly spread on the cell surface (Ciambrone and McKeown-Longo, 1992). Another group also identified uPA and uPAR as being involved in cell adhesion. The adhesion of human myeloid cells provoked by PMA could be abolished by uPA antibodies and reversed by the addition of the ATF-fragment of uPA (Nusrat and Chapman, 1991). Furthermore, PAI-1 was suggested to be responsible for the reduced adherence, but the reason was presumed to be increased PAI-1-uPA turnover (Waltz et al., 1993). While, Waltz and Chapman (1994) subsequently verified vitronectin-uPA dependent adhesion, they did not succeed in characterizing the vitronectin-binding sites (other than integrins) on the cell surface. However, in the same year vitronectin was reported to bind with high affinity to uPAR on cell the surface (Wei et al., 1994b), being further strengthened by concurrent uPA binding to its receptor. Vitronectin-uPAR binding and its reversibility by PAI-1 was demonstrated in human endothelial cells, but this was shown to occur only with soluble vitronectin and there was no involvement of uPAR-vitronectin in substratum-attached vitronectin (Kanse et al., 1996).

Before long it came evident that uPAR is in close contact with integrins, as it could be specifically co-capped with CR3 (CD 11b/18, β_2-integrins) on neutrophil membranes (Wei et al., 1994a) and was co-immunoprecipitated with β_2-integrin antibodies from monocyte lysates (Bohuslav et al., 1995). A few years later, the interaction between uPAR and integrins was confirmed in another study (this particular work focused on β_1-integrins). In addition, it was proved that uPAR modifies integrin function and is found in caveolin-integrin complexes on the cell membranes (Wei et al., 1996). In the same year, uPAR and PAI-1 were reported to have the same binding sites on vitronectin (not identical but overlapping), and thus, PAI-1, which has a higher affinity to vitronectin could compete for uPAR binding on vitronectin (Deng et al., 1996). tPA was also capable of reversing the inhibitory effect of PAI-1 on adhesion. The relative amounts of uPA and

PAI-1 determine whether an adhesive or nonadhesive effects occurs; more PAI-1 favors cell detachment from the substratum, while more uPA supports cell adhesion (Loskutoff et al., 1999).

This picture became even more complicated when another group showed that PAI-1 is capable of inhibiting the migration of smooth muscle cells. This is possible due to sterical hindrance (Stefansson and Lawrence, 1996), since the PAI-1 attachment site on vitronectin overlaps with the binding site of vitronectin on the $\alpha_v\beta_3$-integrin. This was confirmed by others (Kjøller et al., 1997). Yebra et al. (1996) demonstrated that $\alpha_v\beta_5$ was needed for cell migration via uPA-uPAR on vitronectin and not the $\alpha_v\beta_3$-integrin. It has further been demonstrated that in HT-1080 cells, uPAR co-localizes with several integrins, including β_1 and β_3, and it assembles with α_v, α_3, α_5, or α_6; the assembly depends on the composition of the extracellular matrix (Xue et al., 1997). In cell culture, PAI-1 is also found under the cells bound to the substratum. On the substratum, PAI-1 is bound to vitronectin, which in turn originates from the serum, if not produced by the cells. Immobilized PAI-1 can mediate adhesion and spreading of human myogenic cells, and this could be abolished with antibodies against the integrin $\alpha_v\beta_3$ (Planus et al., 1997).

Table 4. Plasminogen activation components and adhesion molecules.

PA Components	Interacting molecules	Adhesive/deadhesive functions
uPAR	VN	adhesion
uPAR+PAI-1	VN	detachment
uPAR+uPA	VN $\alpha_v\beta_3$, $\alpha_5\beta_3$	adhesion
uPAR+uPA +PAI-1	VN $\alpha_v\beta_3$, $\alpha_v\beta_3$	detachment
uPAR+uPA +PAI-1	VN $\alpha_v\beta_3$, $\alpha_v\beta_5$ LRP	detachment integrin inactivation endocytosis
uPAR (uPA?)	$\alpha_4\beta_1$, $\alpha_v\beta_3$ $\alpha_9\beta_1$	cell-cell adhesion

Waltz et al. (1993) proposed that uPA-PAI-1 might cause cell detachment by PAI-1-uPA turnover, but this theory received little attention at that time (Waltz et al., 1993). In

more recent investigations LRP and uPA-uPAR-PAI-1-integrin turnover has been demonstrated involved in cell adhesive/deadhesive functions (Czekay et al., 2001, 2003) (Table 4). The action seems to depend on the amount of uPAR versus integrins. Czekay et al. (2003) demonstrated that PAI-1 detached cells by disrupting the uPAR-vitronectin or the integrin-vitronectin interaction. In both situations, the PAI-1-mediated cell detachment was an uPA-dependent mechanism. They suggested that detachment of cells by PAI-1 depend on the engaged amount of uPA-uPAR-integrin complexes relative to the total amount of active integrins. Furthermore, they revealed that in the PAI-1-mediated detachment of cells, integrins were inactivated first and then the entire adhesion complex (PAI-1-uPA-uPAR-integrin) was internalized via LRP (Czekay et al., 2003). Internalization of the complex occurs via direct interaction between uPAR and LRP (Czekay et al., 2001).

uPAR has been thought to associate with integrins and perhaps modify their functions (Simon et al., 2000), but recently, uPAR has been reported to in fact be a ligand for integrins. uPAR was shown to directly compete with other ligands in binding to integrins (Tarui et al., 2001). In addition, uPAR appears to be a ligand, heterotypic i.e. it binds to neighboring cell integrins.

AIMS OF THE STUDY

This study aimed to gain insight into the mechanisms regulating plasminogen activation.

Specific aims were:

-to study localization, mobility, and associated molecules of the uPAR on the cell surface (I).

-to study regulation of uPAR expression in retinal pigment epithelial cells (II).

-to study the effect of hydrocortisone and other regulatory molecules on the expression of PA genes in normal and immortalized epithelial cells (III).

-to study in vivo expression of tPA in normal, premalignant, and malignant cervical epithelium (IV).

MATERIALS AND METHODS

Cell cultures

Human RD rhabdomyosarcoma cells were from the American Type Culture Collection and human embryonic skin fibroblasts (HES) were established by standard procedures. Both cell lines were cultured in Eagle's minimal essential medium (MEM) supplemented with 8% fetal calf serum (FCS), glutamine, penicillin, and streptomycin. Human retinal pigment epithelial (RPE) cells were obtained from Dr. Michael Boulton, Royal Eye Hospital, Manchester, United Kingdom. The RPE cells were cultured in the presence of 20% FCS, glutamine, penicillin, and streptomycin in Coon's Ham's F12 medium. Human cervical epithelial cells (HCE) transfected with human papillomavirus (HPV-16) DNA (Zheng et al., 1994) were grown in Dulbecco's Modified Eagle Medium (DMEM) and Nutrient MIX-F12 (1:1) supplemented with 2% fetal bovine serum (FBS), streptomycin, penicillin, glutamine, cholera toxin, EGF, hydrocortisone, insulin, and transferrin together with triiodothyronine (T_3).

Normal human bronchial epithelial (NHBE) cells (Clonetics) and adeno-12/SV-40 virus-transformed human bronchial epithelial cells (BEAS-2B) (Reddel et al., 1988) from Dr. Curtis Harris, National Cancer Institute, Bethesda, MD, were grown in bronchial epithelial cell basal medium (BEBM) (Clonetics) supplemented with bovine pituitary extract, hEGF, epinephrine, hydrocortisone, insulin, retinoic acid, T_3, transferrin, and antibiotics.

Immunofluorescence (RD and HES cells)

A live staining method was used for uPAR monoclonal antibodies; i.e. primary uPAR antibodies (Rønne et al., 1991) were applied at ~0°C on cells before any fixation. After fixation with methanol, the cells were stained with rabbit anti-mouse antibodies conjugated with fluorescein isothiocyanate (FITC). For matrix and integrin antibodies, the cells were first fixed with cold methanol, then exposed to primary antibodies, and finally secondary rabbit anti-mouse or donkey anti-rabbit FITC-conjugated antibodies were added.

Double immunofluorescence was carried out at 0°C with uPAR antibodies and then the cells were fixed with cold methanol. After fixation, staining was carried out in the following order: tetramethyl rhodamine isothiocyanate (TRITC) conjugated donkey anti-mouse, rabbit antibodies to vitronectin receptor or vinculin, and FITC-conjugated donkey anti-rabbit IgG. Washing was done at the beginning and end of each incubations and after fixation. Antibodies were diluted in Dulbecco's buffer supplemented with 0.5% BSA.

Antibody-induced clustering (RD and HES cells)

RD and HES cells were grown as mentioned above, except that the cells were treated with uPA or left untreated. Before clustering, uPAR or uPA antibodies were applied at ~0°C. After

primary antibodies, clustering of uPAR/uPA was done with rabbit anti–mouse IgG at 37°C for 15 min. Cell layers were fixed with cold methanol and treated with FITC-conjugated swine anti-rabbit or rabbit anti-mouse IgG.

Immunoferritin electron microscopy (RD cells)

Ultrastructural localization was evaluated by immunoelectron microscopy based on the ferritin-labeling technique. Staining was carried out at 0°C in the following order: primary antibodies (uPAR), rabbit anti-mouse IgG, mouse anti-horse ferritin, and ferritin. After each incubation, the cell layers were washed. Cells were fixed with a mixture of paraformaldehyde and glutaraldehyde in phosphate buffer, after which sodium borohybride was applied. Post-fixation was done with O_sO_4 in phosphate buffer. Cells were then dehydrated with graded series of ethanol, with or without uranyl acetate, at the 94% ethanol stage. Samples were mounted in Epon, after polymerization were sectioned, and sections were stained with lead citrate and uranyl acetate. Samples were observed with a JEOLJEM-1200EX electron microscope.

Flow cytometric analysis (RPE cells)

uPAR protein quantity was determined on the surface of RPE cells. Cells were detached from dishes and washed with 1% serum in PBS. uPAR monoclonal antibodies were applied to cells on ice. After washings, FITC-conjugated anti-mouse IgG was added. FITC-labeled cells were analyzed with FACScan (Becton Dickinson).

EIA for uPAR antigen (RPE cells)

The amount of uPAR was measured from RPE cell lysates. RPE cells were lysed in cold TRIS-buffer containing 1% Triton X-114 and protease inhibitors. Samples were incubated in wells coated with polyclonal uPAR antibodies. After antigen binding, the wells were rinsed and detection was accomplished with the with biotinylated anti-uPAR monoclonal antibodies and avidin-peroxidase method.

RNA extraction and Northern blotting (HCE16/3 and RPE cells)

Guanidium isothiocyanate lysis and phenol chloroform extraction or the CsCl gradient centrifugation method were used to isolate total cellular RNA. RNA was quantified photometrically and samples were fractionated on agarose gel and then transferred to Hybond-N nylon membranes. For nonradioactive detection, hybridization was carried out in the solution containing formamide, sodium citrate (SSC), N-laurosyl sarkosine, sodium dodecyl sulfate (SDS), blocking solution (Boehringer Mannheim), and digoxigenin-labeled probe. For radioactive detection, hybridization was carried out in a solution of formamide, Denhardt's solution, sodium chloride-sodium phosphate EDTA buffer (SSPE), and SDS. After hybridization, filters were washed. Digoxigenin-labeled blots were submitted to immunological detection

using anti-digoxigenin antibody conjugated with alkaline phosphatase, followed by chemiluminescent (CSPD) detection. For radioactively labeled blots, [α-P^{32}]-dCTP and a random-primed DNA labeling kit were used.

Immunocapture assay for plasminogen activators (HCE16/3 and RPE cells)

Multiwell plates were coated with uPA or tPA polyclonal antibodies. Growth medium supernatants and standard dilutions of uPA or tPA enzymes were added to the wells. After incubation, the wells were washed and plasminogen was added to the specific buffer for uPA or tPA. Plasmin substrate (ZLS-Lys-SBzl) was also added to the buffer. uPA and tPA activities were read with a photometer reader at 10-min intervals.

PAI-1 EIA (HCE16/3 cells)

Enzyme immunoassay for PAI-1 was performed in the following order. Multiwells were first coated with mouse anti-human PAI-1. After incubation, the wells were washed and blocked. Samples were then added, and after their incubation, detection was accomplished with biotinylated PAI-1 monoclonal antibody, and peroxidase-conjugated streptavidin and substrate. The reaction was stopped and absorbances of color were read by a multiscanner photometer.

Zymography (HCE16/3 cells)

Samples were run in SDS-PAGE gels under nonreducing conditions, after which the gels were washed thoroughly with Triton X-100 in PBS. Casein gels were made with agarose which contained milk powder and plasminogen in a Tris-HCl buffer, pH 7.4. Washed SDS gels were placed on the top of the agarose gels and incubated at 37°C or at 4°C. Anticatalytic antibodies were added between gels to inhibit plasminogen activators.

Immunoprecipitation of PAI-1 (HCE16/3 cells)

Immunoprecipitation of PAI-1 from the growth medium was achieved with a monoclonal antibody to PAI-1. The immunoprecipitate complex was captured by Protein-A Sepharose, which was pretreated with rabbit anti-mouse IgG. The immunoprecipitate complex was washed and eluted a using nonreducing SDS sample buffer. Immunoprecipitates were separated by SDS-PAGE after which the SDS gels were ready for zymographic analysis.

Tissue specimens

The following 104 archival, formalin-fixed, paraffin-embedded blocks were studied: normal cervical epithelium (n=7), condylomatous atypia (n=13), condyloma acuminatum (n=1), mild dysplasia (cervical intraepithelial neoplasm [CIN I]; n=10), moderate dysplasia (CIN II; n=11) and severe dysplasia or carcinoma in situ (CIN III; n=20) of the dysplastic squamous epithelium, as well as microinvasive epidermoid carcinoma (n=9), invasive epidermoid carcinoma (n=13), adenocarcinoma (n=11), and adenosquamous carcinoma (n=9) of the uterine cervix. All tumor

samples were from primary lesions. Paraffin blocks of tissue specimens were supplied by the Department of Pathology, Helsinki University Central Hospital.

Antibodies and probes

Monoclonal antibodies to human tPA (PAM-3) and uPA (3689) were provided by American Diagnostica (Greenwich, CT) and to human Ki-67, neurofilament protein, and vimentin (clone V9) by DAKO A/S (Glostrup, Denmark). For the RNA in situ hybridization experiments, riboprobes were used. for cDNA fragments; a 2.3-kb PstI fragment of human tPA was from the American Type Culture Collection, Rockville, MD. The templates for riboprobes with either an SP6 or T7 promoter sequence at the 5' end were produced by polymerase chain reaction. The probe size was 243 bases for tPA (position 1431-1674) and was labeled with digoxigenin-uridine triphosphate by in vitro transcription. In negative control hybridization, probes in sense orientation were used.

Immunohistochemical detection of tPA

Immunohistochemistry of tPA was done by using a Ventana Gen II automated immunohistochemical stainer (Ventana Medical System, Tucson, AZ). Three micromillimeter thick sections on SuperFrost Plus slides (Menzel-Gläser, Braunschweig, Germany) were assayed for tPA and neurofilament antigens using the Ventana Basic Alkaline Phosphatase Red detection kit. For Ki-67 and vimentin antigens Ventana Basic DAB detection kit was used. The kits included a universal biotinylated secondary antibody. The sections were deparaffinized and hydrated. Tissue slides were pretreated in a microwave oven, cooled with distilled water, and placed in a Ventana Tris-based APK buffer (Ventana Medical Systems). The instrument was programmed for a 28-min incubation with primary antibody, a 4-min postfixation, and incubation with biotinylated secondary antibodies. The complex was visualized by avidin alkaline phosphatase conjugate (RED), avidin horseradish peroxidase (DAB) and followed by alkaline phosphatase substrate Fast Red, peroxidase substrate diaminobenzidine (DAB). Enhancement was done with magnesium chloride in the Red and copper sulfate in the DAB detection kit. The program included counter-staining with hematoxylin and post-counter-staining with Ventana Bluing Reagent. Sections were dehydrated with ethanol and xylene and mounted with Entellan®-treated coverslips.

Semiquantitative scoring of tPA level was done visually: tissues were scored using 3 for dark red staining (strong), 2 for medium red staining (moderate), 1 for weak but positive staining (mild), and 0 for negative or very faint staining.

In situ hybridization

ISH was performed using the Ventana Gen II Slide Stainer. The program used was the "Ventana Regular" protocol. Sections were first deparaffinized and rehydrated in graded series

of ethanol. After rinsing in distilled water treated with 0.1% diethylpyrocarbonate (DEP), the slides were kept in APK buffer until the in situ hybridization procedure. The sections were treated for 8 min with protease before hybridization at 45°C for 14 h. The hybridization mixture contained 50% formamide and 4 x SSC. Three washes after hybridization were performed at 65°C for 8 min in 1 x SSC, 0.5 x SSC, and 0.1 x SSC, respectively. Monoclonal anti-digoxigenin clone DI-22 (Sigma, St Louis, MO) was incubated with the sections for 28 min. The probe was detected with the Ventana 3,3'-diaminobenzidine tetrahydrochloride (DAB) biotin avidin detection kit. The sections were dehydrated and mounted with coverslips treated with Entellan® and then analyzed with an Olympus light microscope.

Immunohistochemistry and in situ hybridization results were photographed with a Zeiss Axiophot 2 image automated microscope.

RESULTS AND DISCUSSION

Localization and lateral mobility of uPAR and effect of TGF-β on uPAR expression (I, II)

The distribution of uPA reflects the uPAR distribution on cell membranes (Pöllänen et al., 1988). We confirmed that uPAR is also found at the focal contacts, tips of macrospikes, lamellipodia, and cell-cell contacts of human rhabdomyosarcoma and embryonic fibroblasts (I). However, in some cell lines, mostly epithelial, uPAR has not been found in focal contacts. In Met24 melanoma cells, for instance, it was distributed in a punctate pattern and localized in caveolae (Stahl and Mueller, 1995). In human amnion WISH cells, uPAR was also localized in a punctate pattern all over the cell membranes (Busso et al., 1994).

We have also observed that uPAR in a punctate pattern on plasma membranes of human amnion epithelial cells. This was especially the case with confluent cells, because in sparse and scattered cell cultures, uPA was found in focal adhesion plaques on plasma membranes (unpublished results). uPAR can be differentially distributed depending on cell type, but distribution may also depend on the condition of cells: i.e. whether they are in an adhesive state or in a migratory or stationary state. Moreover, in study I, uPAR in migratory RD cells gave a spot-like staining on the cell surface and not an elongated striae-like pattern.

Colocalization with uPAR was found with $\alpha_v\beta_3$-integrins (I). It has subsequently been reported that uPAR colocalizes with numerous integrins, e.g. with $\alpha_v\beta_5$- $\alpha_v\beta_3$- and $\alpha_3\beta_1$-integrins, depending on the cell type studied (Yebra et al., 1996; Planus et al., 1997; Carriero et al., 1999; Ghosh et al., 2000).

Ciambrone and McKeown-Longo demonstrated in 1992 that vitronectin is responsible for clustering of uPA to the focal adhesion contacts on fibrosarcoma cells, although they later reported that vitronectin and its $\alpha_v\beta_5$-receptor were necessary but not sufficient to cluster uPA to focal contacts (Wilcox-Adelman et al., 2000). Integrins affect plasminogen activation in many ways. They not only target uPAR/uPA on the plane of the plasma membrane but also influence the expression of uPA/uPAR (Ghosh et al., 2000; Hapke et al., 2001; Silvesteri et al., 2002). We found uPA to be one (perhaps not the only) candidate participating in the driving or anchoring of uPAR to focal contacts on the cell

membrane (I). This was based on the unoccupied receptor being diffusely distributed, while the occupied receptor was mainly bound to restricted areas on fibroblast cell membranes (I). In addition, in RD rhabdomyosarcoma cells, which secrete high amounts of uPA, uPAR was mainly located in these restricted areas (focal adhesions) (I). Furthermore, antibody-induced clustering suggested that uPAR, which was localized at the focal contacts, was not laterally mobile (Fig. 3). Clusters were seen within the focal contacts in both fibroblasts and RD cells.

We detected uPAR under cell-cell adhesion sites (I). This was seen as a continuous line between adjacent adhering cells at the light microscopic level. In the electronmicroscopic study, uPAR was seen organized focally between adjacent cells (I). Subsequent studies by others have shown an interaction of uPAR with integrins on the adjacent cells (Tarui et al., 2001).

In amnion WISH cells, the addition of exogenous uPA in serum-free conditions had no effect on the distribution of uPAR (Busso et al., 1994), although cell morphology was affected. Moreover, uPA was reported to not necessarily be required for uPAR localization to focal contacts on the human glioma cell surface (Hedberg et al., 2000). This was based on the finding that uPAR was detected in focal contacts when the cells were cultured in the presence of serum or certain serum fractions and on glioma cells being known not to produce uPA. Glioma cells were concluded not to need “classical focal adhesions” for their migration, nor was uPAR needed in the focal adhesions of these cells to migrate. Moreover, the authors suggested that uPAR could preferentially be an adhesion receptor in glioma cells because the presence of uPAR at the focal adhesions correlated with cell spreading rather than with elongated cell morphology. They also demonstrated that high molecular weight components of serum could cause the localization of uPAR to focal contacts.

Although uPAR/uPA-PAI-vitronectin and its receptor take part in cell adhesion and cell migration, they (uPAR, uPA, PAI-1) are an additional mechanism; they are not obligatory for cells to be able to adhere or migrate.

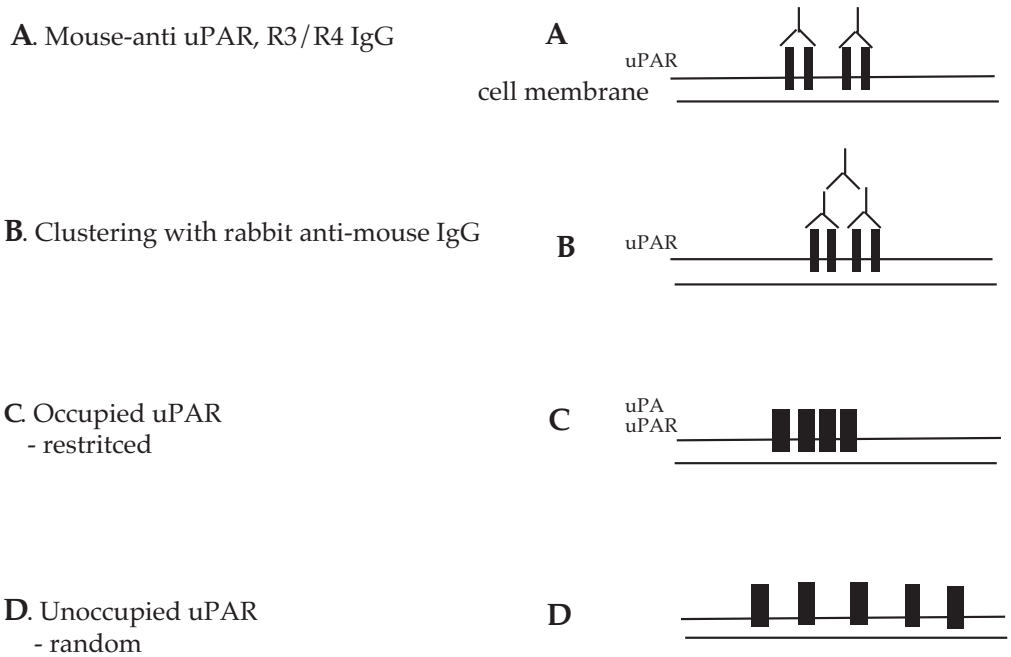

Figure 3. A model of in vitro and in vivo induced distribution of uPAR on the cell surface. Occupied uPAR is clustered at focal contacts (C), and unoccupied receptors have a uPAR punctated pattern all over the cell membrane (D).

TGF-β induced rapid and transient uPAR mRNA expression in retinal pigment epithelial (RPE) cells (II). This was seen in the uPAR protein expression level at 48 h on cell membranes. The appearance of uPAR protein in the cell lysate and on the cell membranes reflects a transient effect of TGF-β on uPAR production by these cells. The highest amount of uPAR in cell lysates was measured at 24 h, with a decrease occurring thereafter. Induction of uPAR release by TGF-β could also be seen in the growth medium. Interferon-γ (IFN-γ) had an increasing effect on uPAR gene transcription, but this was not translated to the protein level (II). In fact, a decrease occurred in uPAR protein levels of RPE cells with the addition of interferon. Some reports claim that in human monocytes IFN-γ increased uPAR, but addition of uPA together with IFN-γ decreased the number of uPA-binding sites on the cell surface (Kirchheimer et al., 1988).

uPA can increase its own or uPAR expression in bronchial epithelial cells (Idell, 2002). IFN-γ, as well as IFN-α, has been shown to regulate plasminogen activation by decreasing PAI-1 expression, resulting in increased uPA activity (Sirén et al., 1994). uPAR mRNA turnover may also be regulated by IFN-γ. Cytokines and growth factors expressed in such eye diseases as PVR and ARMD influence the development and severity of these diseases. IL-1 enhances uPAR expression as well as collagenolytic activity with increased RPE cells invasion throughout extracellular matrix. RPE cell invasion could be abolished by PAI-1 but not by collagenase inhibitor (1,10-phenantroline). Nevertheless, PAI-1 and collagenase inhibitor were shown to be able to inhibit proteolysis of extracellular matrix (Elner, 2002). A recent study concluded that uPA, tPA, and plasminogen, but not uPAR, were involved in CNV which is severe form of ARMD. All PA mRNA components were expressed in human CNV patient specimens (Rakic et al., 2003). The same study showed that tPA-, uPA-, and plasminogen-deficient mice were resistant to choroidal neovascularization. Previous reports have also indicated that new vessel formation is inhibited in PAI-1-deficient mice (Lambert et al., 2001). Our results demonstrate that TGF-β can disrupt the balance of plasminogen activation by increasing uPAR expression. Increased uPAR may have an enhancing effect on proteolytic activity, but it also influences the migratory behavior of RPE cells.

Hydrocortisone has a strong impact on plasminogen activators (III)

Hydrocortisone was found to be a potent repressor of plasminogen activators in the HCE16/3 cervical epithelial cell culture system. Plasminogen activators and their inhibitor PAI-1 were studied at the protein and RNA expression levels. We also evaluated the effect of hydrocortisone on plasminogen activators in two additional cell lines, namely BEAS-2B (adeno/Sv-40 hybrid transfected and immortalized bronchial epithelial cells) and its normal counterpart NHBE (normal human bronchial epithelial) cells. The main finding was that tPA was under the strong repressive control of hydrocortisone. Its gene expression started to increase 48 h after the withdrawal of hydrocortisone. By contrast, uPA gene induction was seen as early as 3 h after withdrawal of hydrocortisone. None of the other growth supplements in the conventional epithelial cell culture medium, including 2% serum (FBS), EGF, insulin,

cholera toxin, and transferrin together with T_3, had such an effect on tPA production in HCE16/3 cells. EGF increased the expression of uPA in these cells.

Earlier studies have revealed that both activators respond to glucocorticoids and that the extent of the effect seems to depend on cell type. Recent publication states that the tPA gene has four glucocorticoid receptor (GR) binding sites that act as enhancers as well as cooperating with a retinoic acid (RA) response element (Bulens et al., 1997). GR binding sites have thus far not been found in the uPA gene structure. However, uPA does have has AP-1 binding sites, and GR repressive function has been shown to go through AP-1 transcription factors (De Cesare et al., 1995).

Both PAI-1 and tPA plasma levels display circadian rhythm, the reason for which remains unknown. The tPA activity is lowest early in the morning and increases during the day, while PAI-1 activity is highest in the morning (Wiman, 1995). They both are regulated by glucocorticoids and contain glucocorticoid receptor response elements (GRE) in their genes. Thus, it is worth noting that cortisol also displays a circadian rhythm. In human plasma, cortisol is in its maximum level early in the morning, declining throughout the day (Brook and Marshall, 1999).

Expression of tPA in the stratified squamous epithelium of human uterine cervical tissues (IV)

Based on our previous study (III), we were interested in investigating tPA antigen and mRNA expression. We showed that tPA is produced in normal, dysplastic stratified epithelium as well as malignant tissues of the human uterine cervix. Intense tPA antigen staining was localized in the 3-4 cell layers above the basal cells of the stratified squamous epithelium (IV). This was seen both normal, dysplastic (CIN I-III) and microinvasive epidermoid carcinoma specimens. Normal, condyloma and CIN I uterine cervical epithelium mostly gave strong staining intensity, however, in CIN II and CIN III dysplastic tissue specimens there was a tendency for decreased staining intensity along with tumor progression. The vessel wall of the endothelial cells and granulocytic blood cells within the vessels were tPA antigen-positive (IV).

An earlier study has shown tPA mRNA to be localized mainly in the middle layers of dysplastic stratified squamous epithelium of the human uterine cervix (Riethdorf et al., 1999). Our results indicated the presence of tPA mRNA in the basal and parabasal layers of the stratified epithelium (IV). In addition, another group has reported that tPA

antigen was expressed from the basal to the upper layers of the stratified epithelium in precancerous lesions (Larsson et al., 1987). We could not confirm this. tPA immunoreactivity mostly was evident starting from a 3-4 layers above the basal cell layer in the stratified epithelium (IV).

In Epidermoid carcinoma tissue specimens, tPA immunoreactivity was mostly at a strong to moderate level of intensity. By contrast, in adenocarcinoma specimens, staining gave mostly weak and negative color intensity. Two of the adenocarcinoma patient specimens exceptionally showed a very strong staining of tPA antigen. For these specimens, in situ hybridization was performed. Interestingly, this showed a strong but heterogeneous localization of tPA mRNA. tPA mRNA was clearly expressed in a certain population of cancer cells. Heterogeneous distribution was also detected at the antigen level in the same specimens. tPA mRNA was found in adenocarcinoma cancer cells but not in epidermoid carcinoma samples. However, around the epidermoid carcinoma, the stromal cells were tPA mRNA-positive. tPA antigen was also detected in stromal cells. The cells, which displayed the positive signal, were inflammatory and fibroblast-like. Around the cancer cell islands, positive tPA immunostaining was frequently seen. Low tPA antigen or activity levels have been shown to predict poor prognosis in breast cancers. In the case of colorectal cancer, uPA, uPAR and PAI-1/2 levels are elevated, but tPA decreases with progressing carcinogenesis. A decrease in tPA is seen especially when compared with the corresponding normal mucosa. High tPA antigen and activity levels in normal mucosa correspond to good prognosis of patients (Ganesh et al., 1994; Verspaget et al., 1995). High tPA expression detected by immunohistochemistry in the cancer cells of primary melanoma predicts a long metastasis-free interval (Ferrier et al., 2000).

tPA is apparently necessary for cancer invasion, growth and angiogenesis. In humans, this has been demonstrated pancreatic carcinomas, oral squamous cell carcinoma, and in neuroblastoma cell lines (Tiberio et al., 1997; Uchida et al., 2001; Díaz et al., 2002). In human pancreatic cancer cell lines, tPA was overproduced in well-differentiated cancer cells together with uPAR (Paciucci et al., 1998). In oral squamous and neuroblastoma cells, RA enhances tPA activity and invasion of cancer cells, which is accompanied by proMMP-2 and -9 activation in oral cell carcinomas (Tiberio et al., 1997; Uchida et al., 2001).

We found tPA mRNA and antigen to be located in different layers in the stratified squamous epithelium. The method used for in situ hybridization did not give any background, accurate visualization of tPA mRNA was achieved. tPA may have additional roles to that of being a fibrinolytic enzyme in normal epithelial cells. Plasminogen activators have been linked to or suggested to play a role in cell differentiation by many investigators (Wilson and Francis, 1987; Neuman et al., 1989; Jensen et al., 1990; Chen et al., 1993). Secretion of tPA is a differentiation marker in embryonal carcinoma cells which can be induced to differentiate by retinoic acid (Strickland et al., 1980; Watkins et al., 1992). Normal urothelium secretes uPA and tPA, but tumor cell lines of urothelium secrete only uPA (Dubeau et al., 1988). Results from an immunohistological study have suggested that tPA might be a marker of "end-stage differentation" in vivo (Dubeau et al., 1988). However, the targets for plasminogen activators/plasmin in differentiating normal cells remain unclear. Some studies have indicated that adhesive molecules, such as fibronectin and fibrinogen/fibrin, hinder the cell differentiation process, and this could be overcome by activation of plasminogen (Selvarajan et al., 2001; Suelves et al., 2002). In adipocyte differentiation, kallikrein was shown to activate plasminogen but not tPA or uPA (Selvarajan et al., 2001).

Our study revealed that tPA is produced, possibly in large amounts in human normal and dysplastic stratified cervical epithelium. While tPA expression may be turned off in cancer cells with cervical tumor progression, we detected tPA antigen in most of the epidermoid carcinoma specimens. This could be due to paracrine secretion of tPA from the surrounding stromal cells. Studies of tPA in the brain and in some cancer cells have indicated that tPA has another role in addition to fibrinolysis. Intrestingly, tPA, like uPA, also appears capable reversing PAI-1 cell detachment. To date, this has not, however, prompted research into an association between tPA and cell adhesion. uPA is known to be able to function without its receptor and tPA has been established to have high intrinsic activity in its zymogen form and to efficiently catalyze plasminogen activation in the presence of co-factors such as fibrin or other binding proteins. It is therefore justified to assume that tPA via plasmin or itself, might have a role in normal epithelial cells. In addition, future studies should investigate the regulation of tPA with tumor progression. Two of plasminogen activators, uPA and tPA, appear to be regulated in opposite ways in certain cancers.

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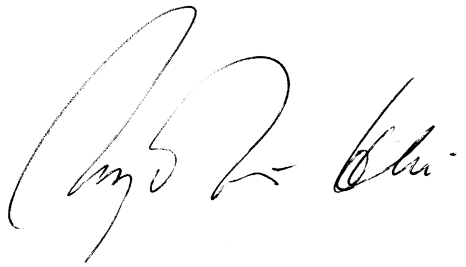

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